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Report

A Molecular Framework of Light-Controlled Phytohormone Action in *Arabidopsis*

Shangwei Zhong,[1,2,5] Hui Shi,[1,3,5] Chang Xue,[1] Lei Wang,[1,2] Yanpeng Xi,[1] Jigang Li,[1,2] Peter H. Quail,[3] Xing Wang Deng,[1,2,*] and Hongwei Guo[1,4,*]

[1]Peking-Yale Joint Center for Plant Molecular Genetics and Agro-Biotechnology and State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing 100871, China

[2]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520, USA

[3]Department of Plant & Microbial Biology, University of California, Berkeley, Berkeley, CA 94720, USA

[4]Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China

Summary

Environmental changes strongly affect plant growth and development. Phytohormones, endogenous plant-made small molecules such as ethylene, regulate a wide range of processes throughout the lifetime of plants [1, 2]. The ability of plants to integrate external signals with endogenous regulatory pathways is vital for their survival [3, 4]. Ethylene has been found to suppress hypocotyl elongation in darkness [5] while promoting it in light [6, 7]. How ethylene regulates hypocotyl elongation in such opposite ways is largely unknown. In particular, how light modulates and even reverses the function of ethylene has yet to be characterized. Here we show that the basic-helix-loop-helix transcription factor phytochrome-interacting factor 3 (PIF3) is directly activated by ETHYLENE-INSENSITIVE 3 (EIN3) and is indispensible for ethylene-induced hypocotyl elongation in light. Ethylene via EIN3 concomitantly activates two contrasting pathways: the PIF3-dependent growth-promoting pathway and an ethylene response factor 1 (ERF1)-mediated growth-inhibiting pathway. In the light, growth-promoting PIFs are limiting due to light-dependent destabilization, and thus ethylene stimulates growth under these conditions. In contrast, ERF1 is destabilized, and thus limiting, under dark conditions, explaining why ethylene inhibits growth in the dark. Our findings provide a mechanistic insight into how light modulates internal hormone-regulated plant growth.

Results and Discussion

PIF3 Acts Downstream of EIN3/EIL1 in Mediating Ethylene-Stimulated Hypocotyl Elongation in Light

It has been uncovered that in the ethylene signaling pathway, the receptors and CTR1 are upstream repressors, whereas ETHYLENE-INSENSITIVE 2 (EIN2), EIN3, and EIN3-like 1 (EIL1) are downstream activators [2, 8, 9]. Previous studies showed that the ethylene-insensitive mutant *ein2* exhibits shorter hypocotyls whereas the constitutive ethylene response mutant *ctr1* displays longer hypocotyls than wild-type (WT) seedlings grown in light [6, 7]. We found that the light-grown ethylene-insensitive mutant *ein3 eil1*, which lacks both EIN3 and EIL1 transcription factors, also displayed shortened hypocotyls (see Figures S1A and S1B available online), whereas transgenic plants overexpressing EIN3 (EIN3OX) constitutively exhibited elongated hypocotyls compared with Columbia-0 WT (Col-0) (Figures S1A and S1B). Moreover, treatment with 1-aminocyclopropane-1-carboxylic-acid (ACC), a biosynthetic precursor of ethylene, promoted hypocotyl elongation of light-grown WT seedlings but not *ein3 eil1* mutant seedlings (Figures 1A and S1C). Therefore, EIN3 and EIL1 are required for ethylene-induced hypocotyl elongation in light.

Recent studies have revealed that phytochrome-interacting factors (PIFs) play central roles in repressing photomorphogenesis [10–12]. Because ethylene-induced hypocotyl elongation antagonizes light-regulated plant development, we investigated whether PIFs are involved in ethylene-induced hypocotyl elongation. Strikingly, upon ACC treatment, although *pif1*, *pif4*, and *pif5* mutants displayed no obvious difference from WT, the *pif3* mutant was almost entirely impaired in ethylene-induced hypocotyl elongation, mimicking the *ein3 eil1* mutant (Figures 1A and S1C). A dosage-response experiment further confirmed that *pif3* was virtually insensitive to ACC in the regulation of hypocotyl elongation (Figure 1B). Microscopic observation demonstrated that ethylene promoted hypocotyl length mainly by inducing cell elongation, which was abolished in the *pif3* mutant (Figure 1C). These results indicate that PIF3 is an essential component required for ethylene-induced hypocotyl elongation in light.

We next assessed the genetic relationship between these two classes of transcription factors, EIN3/EIL1 and PIF3. Overexpression of PIF3 (PIF3OX) led to robust hypocotyl elongation even in the absence of ethylene, and it completely rescued the shortened hypocotyls of *ein3 eil1* (Figures 1D and S1D). Conversely, *pif3* fully suppressed the elongated hypocotyls of *ctr1*, whereas *pif3 ein3 eil1* triple mutants displayed the same short-hypocotyl phenotype as *pif3* and *ein3 eil1* (Figures 1D and S1D). These genetic interactions suggest that PIF3 acts in the same pathway downstream of EIN3 and EIL1 in regulating ethylene-promoted hypocotyl elongation in light.

EIN3 Activates *PIF3* Gene Expression by Directly Binding to the Promoter Elements of *PIF3*

To reveal how EIN3 and EIL1 regulate PIF3, we first examined whether ethylene regulates *PIF3* transcription via EIN3 and EIL1 in light. Upon ACC treatment, *PIF3* transcription was notably elevated in WT, but not in the *ein3 eil1* mutant (Figure 2A). Consistently, *PIF3* transcript levels were almost eliminated in *ein2* and *ein3 eil1* but drastically increased in *ctr1* (Figure 2A). In addition, we also examined *PIF3* transcription in transgenic plants expressing EIN3 protein under control of the estradiol-inducible promoter in the *ein3 eil1 ebf1 ebf2* quadruple-mutant background (*iE/qm*) [13]. *PIF3* expression was upregulated rapidly (in 10 min) and in a dosage-dependent manner following the induction of EIN3 protein in *iE/qm* seedlings (Figure 2B). Notably, the patterns of EIN3-induced *PIF3*

[5]These authors contributed equally to this work

*Correspondence: xingwang.deng@yale.edu (X.W.D.), hongweig@pku.edu.cn (H.G.)

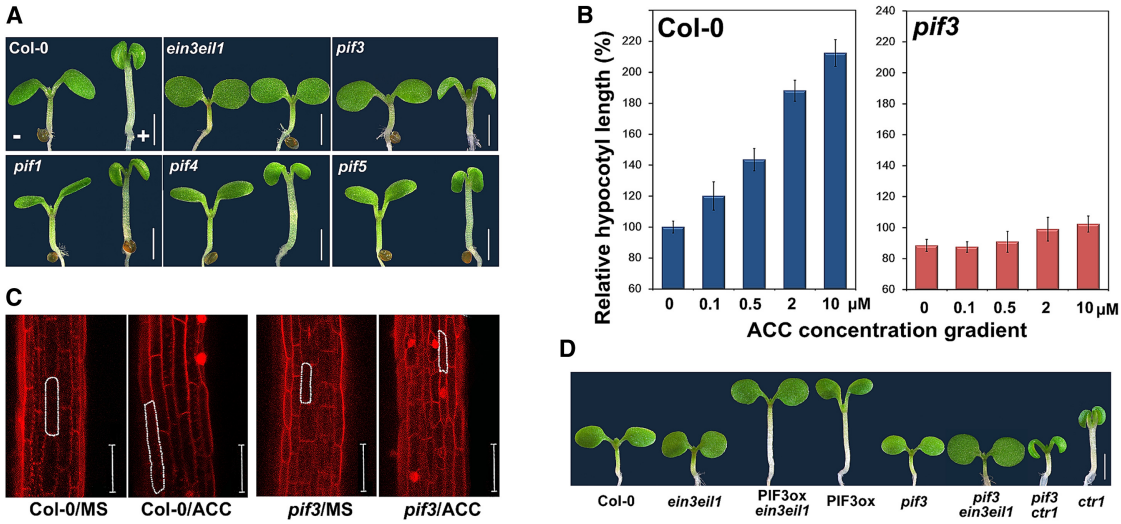

Figure 1. PIF3 Acts Downstream of EIN3 and EIL1 in Mediating Ethylene-Stimulated Hypocotyl Elongation in Light

(A) Phenotype of 5-day-old light-grown seedlings on MS medium supplemented with (+, right) or without (−, left) 10 μM 1-aminocyclopropane-1-carboxylic-acid (ACC).

(B) Hypocotyl lengths of 7-day-old light-grown seedlings on MS supplemented with increasing concentrations of ACC. Data are shown as mean ± SD; n > 20.

(C) Propidium iodide staining of hypocotyl cells of 7-day-old light-grown seedlings.

(D) Hypocotyl phenotype of 5-day-old light-grown seedlings on MS.

See also Figure S1.

expression were similar to those of *EBF2* and *ERF1*, two known direct target genes of EIN3 (Figure S2A) [14, 15]. By contrast, transcript levels of *PIF1*, *PIF4*, or *PIF5*, which play no role in ethylene-induced hypocotyl elongation, were not significantly regulated by EIN3/EIL1 or ethylene (Figure S2B). GUS staining of *PIF3*p:GUS transgenic plants further demonstrated that the *PIF3* promoter was active in cotyledons and hypocotyls, where its activity was markedly enhanced by ACC treatment or in the *ctr1* mutant but clearly decreased in the *ein3 eil1* mutant (Figure 2C). Therefore, ethylene stimulates *PIF3* gene expression via the action of EIN3 and EIL1.

The similar kinetics between the induction of *PIF3* and that of *EBF2* and *ERF1* suggested that *PIF3* might be a direct target gene of EIN3 (Figures 2B and S2A). Previous studies showed that EIN3 binds to specific promoter elements named EIN3 binding sites (EBSs) to regulate downstream gene expression [15, 16]. Bioinformatics analysis identified several putative EBSs in the *PIF3* promoter (Figure S2D). We thus divided the *PIF3* promoter sequence into four regions, A through D, each containing at least one putative EBS element (Figure 2D). Chromatin immunoprecipitation of light-grown *iE/qm* seedlings followed by PCR assay showed that EIN3 preferentially binds to regions A, B, and C of the *PIF3* promoter in vivo (Figures 2D and S2C). Subsequent yeast one-hybrid assay revealed that EIN3 binds specifically to region B in yeast cells (Figure S2E), implying that additional factors might be required to enable EIN3 association with regions A and C in planta. Further yeast one-hybrid assays using mutated EBS elements showed that one of the three putative EBS elements in region B (CTCTGC) primarily mediates EIN3 binding in yeast (Figures S2D and S2E). Electrophoretic mobility shift assay indicated that EIN3 was able to directly bind to the three EBS elements in the region B in vitro (Figures 2E and 2F). Moreover, ACC induction of *PIF3*p:GUS activity was greatly decreased when all three EBS elements in region B of the *PIF3* promoter were mutated (Figure 2H), further confirming the essential role of these EBS elements in mediating ethylene-activated *PIF3* gene expression. Taken together, these results indicate that EIN3 directly binds to the specific EBS elements in the *PIF3* promoter to activate its transcription.

Ethylene Activates a PIF3-Dependent Growth-Promotion Pathway that Is Progressively Fortified with Light-Induced PIF Degradation

Interestingly, we found that, as in the light condition, ethylene also induced *PIF3* gene expression in darkness in an EIN3- and EIL1-dependent manner (Figure S3A). We then examined whether PIF3 contributes to ethylene-regulated hypocotyl elongation in dark-grown seedlings. In contrast to their pronounced phenotypes in light, neither *pif3* mutants nor PIF3OX showed any obvious difference from WT with or without ACC treatment of etiolated seedlings (Figure 3A). The finding that PIF3 seems to lack a role in hypocotyl growth in dark could be attributed to functional redundancy among multiple PIFs. Previous studies showed that PIF1, PIF3, PIF4, and PIF5 are highly abundant and act redundantly to promote hypocotyl elongation in dark, because none of the single, double, or triple mutants but only the *pif1 pif3 pif4 pif5* quadruple mutant (*pifQm*) displays significantly reduced hypocotyls [10, 11] (Figure S3B). As reported previously [10], our results demonstrated that all of the PIF1, PIF3, PIF4, and PIF5 proteins accumulated in darkness and dramatically declined upon light irradiation (Figure S3C). We found that with ACC treatment, light-induced degradation of the constitutively expressed PIF3-MYC protein was different from that on MS (Figure S3C), suggesting a possible role of ethylene in affecting PIF3 protein stability. However, light predominantly degrades PIF proteins (Figure S3C). It is thus likely that PIF functions have reached a saturated level in etiolated seedlings and that the ethylene induction of PIF3 or overexpression of PIF3 by genetic means

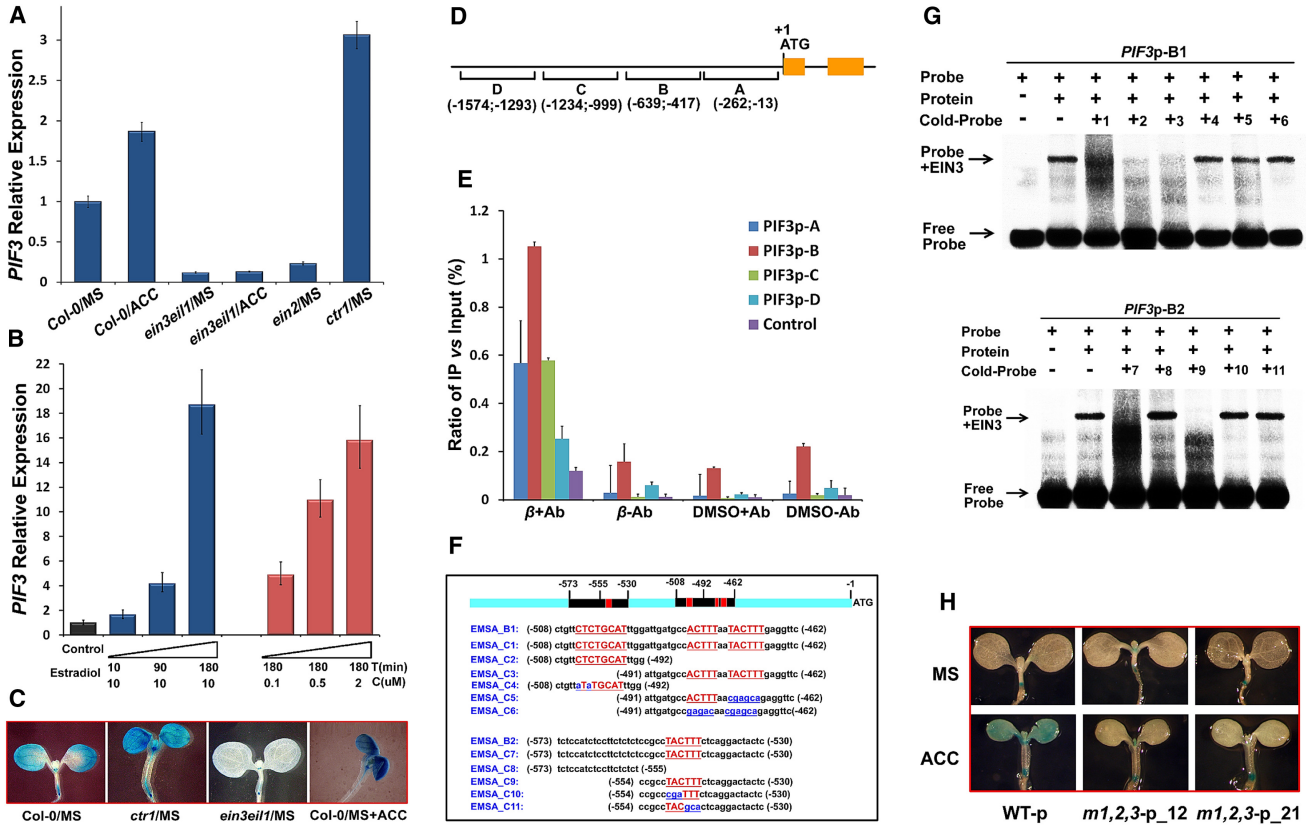

Figure 2. EIN3 Activates *PIF3* Gene Expression by Directly Binding to the Promoter Elements of *PIF3*

(A) qRT-PCR analysis of *PIF3* gene expression in 5-day-old continuous light-grown seedlings on MS (MS) or MS supplemented with 10 μM ACC (ACC). Data are shown as mean ± SD; n = 3.

(B) qRT-PCR analysis of *PIF3* gene expression in 5-day-old continuous light-grown seedlings on MS medium with gradually induced EIN3 protein synthesis. Data are shown as mean ± SD; n = 3. Seedlings expressing estradiol-inducible EIN3 protein in an *ein3 eil1 ebf1 ebf2* mutant background (iE/*qm*) were treated with estradiol at gradient concentrations (C) or treatment time (T).

(C) GUS staining of 5-day-old continuous light-grown *PIF3* promoter:GUS seedlings.

(D) Diagram of the four *PIF3* promoter regions, named A–D.

(E) Chromatin immunoprecipitation results using anti-FLAG antibody (±Ab) to first precipitate the induced EIN3-FLAG protein (β indicates estradiol-induced; DMSO indicates uninduced control) followed by qPCR detection of the four *PIF3* promoter regions in 5-day-old continuous light-grown iE/*qm* seedlings. Data are shown as mean ± SD; n = 3.

(F) Sequences of the probes used in (G). Bases in red underlined capital letters indicate the predicted EIN3 binding motifs; the base changes in the mutant sequences are shown in blue lowercase letters.

(G) Electrophoretic mobility shift assay of recombinant EIN3 protein (aa 1–314) and *PIF3* promoter regions. Cold probes (unlabeled probes) used for competition were in 200-fold excess over labeled probes.

(H) GUS staining of 5-day-old continuous light-grown *PIF3* promoter:GUS transgenic seedlings. WT-p indicates intact *PIF3* promoter fused with GUS reporter gene; *m1,2,3*-p_12 and *m1,2,3*-p_21 indicate two individual transgenic lines of the *PIF3* promoter with all three EIN3 binding motifs mutated.

See also Figure S2.

cannot exert an additive effect on promoting hypocotyl elongation in dark. Nevertheless, when grown in light, most PIF proteins are degraded, leading to low abundance of PIF proteins (Figure S3C) [10, 12]. Under this condition, the action of individual PIFs becomes discernible, as evidenced by the findings that the *pif3*, *pif4*, and *pif5* monogenic mutants each displayed shorter hypocotyls than WT and that ethylene-promoted hypocotyl elongation was eliminated by the *pif3* single mutant (Figure S3D).

To address this scenario in more detail, we measured the effect of ethylene on hypocotyl elongation in a series of photoperiods ranging from complete darkness to continuous light. In WT seedlings, ethylene dramatically repressed hypocotyl elongation in dark, but it gradually turned into a stimulatory signal with increasing light duration (Figure 3B). As expected, *ein3 eil1* completely abolished any response to ethylene treatment regardless of the photoperiod conditions (Figure 3B). However, *pif3* was found to behave like WT in the short-photoperiod conditions under which ethylene was repressive but entirely failed to respond to ethylene in the long-photoperiod conditions where ethylene was stimulatory for hypocotyl elongation (Figure 3B). The influence of light intensity on ethylene-regulated hypocotyl elongation was also examined. Similarly, ethylene's effect on hypocotyl elongation was progressively switched from repressive to stimulatory with increasing intensity of continuous light (Figure 3C). Likewise, *pif3* behaved like WT under weak light intensities (<10 $\mu mol\ m^{-2}\ s^{-1}$) but behaved like *ein3 eil1* under strong light intensities (>20 $\mu mol\ m^{-2}\ s^{-1}$) (Figure 3C). Therefore, EIN3 and EIL1 are required for the dual actions of ethylene in hypocotyl elongation, repression in dark and stimulation in light, whereas PIF3 only mediates ethylene's stimulatory function in light.

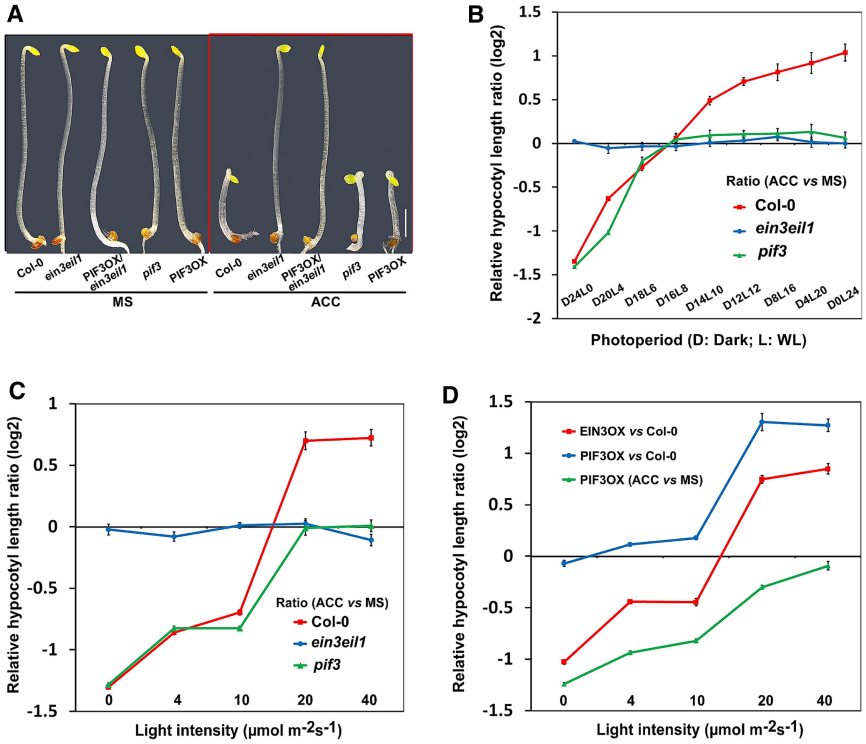

Figure 3. Ethylene-Activated PIF3 Pathway Is Progressively Fortified with Light-Induced PIF Protein Degradation

(A) Hypocotyl phenotypes of 3-day-old seedlings grown on MS ± 10 μM ACC.

(B–D) Ratios (in log2) of hypocotyl lengths of 5-day-old seedlings under increasing photoperiods (130 μmol m^{-2} s^{-1}) (B) or intensities of continuous light (C and D) on MS ± 10 μM ACC. Data are shown as mean ± SD; n > 20.

See also Figure S3.

Further support for this notion came from observations that, with increasing light intensity, the effect of EIN3OX on hypocotyl elongation was gradually reversed from repression to stimulation, whereas PIF3OX displayed little effect on hypocotyl elongation in dark but became progressively more effective in light (Figure 3D). Therefore, with increasing light intensity, PIF redundancy is gradually dampening and ethylene-induced PIF3 becomes progressively more effective in modulating hypocotyl elongation.

Ethylene Activates an ERF1-Dependent Growth-Inhibition Pathway that Is Gradually Saturated by Light

Ethylene application further decreased the hypocotyl length of the *pifQm* mutant in dark (Figure S3B), implying that ethylene inhibits dark-grown hypocotyl elongation through a separate pathway independent of PIFs. Furthermore, the hypocotyl of PIF3OX was notably inhibited by ACC treatment in dark and dim light, and such ACC-evoked inhibition was progressively attenuated with increasing light intensity (Figure 3D). These results suggest the existence of a PIF3-independent pathway mediating ethylene-inhibited hypocotyl elongation even when the function of PIFs is saturated.

We next sought to further characterize the molecular composition of the ethylene-induced growth-inhibition pathway. Of the ethylene-regulated genes, ethylene response factor 1 (ERF1), an AP2-type transcription factor, represented a good candidate because it was previously reported to be a direct target of EIN3 and to suppress hypocotyl elongation [15]. We found that ethylene activated *ERF1* gene expression in both dark and light conditions in an EIN3/EIL1-dependent manner (Figures S2A and S4A). Moreover, transgenic overexpression of ERF1 (ERF1OX) led to marked inhibition of hypocotyl elongation in dark (Figure S4B), mimicking the effect of ethylene. However, this inhibition gradually diminished with the increase of light intensity and eventually disappeared in strong light conditions (Figures 4A and 4B), reminiscent of the inhibitory effect of ethylene on PIF3OX (Figure 3D). Therefore, ERF1 mediates an ethylene-activated growth-inhibition pathway that operates effectively in dark and dim light but hardly in strong light conditions.

Light Destabilizes PIF3 but Stabilizes ERF1 to Control the Output of Ethylene's Effect on Hypocotyl Elongation

We then investigated how light modulates the function of ERF1. In sharp contrast to PIF proteins, we found that ERF1 protein was evidently stabilized when transferring the dark-grown seedlings to light exposure (Figure 4C). Conversely, ERF1 protein became unstable when light-exposed seedlings were returned to darkness (Figure 4D). ERF1 belongs to a large family of ERFs, most of which contain a highly conserved GCC-box binding domain, and some ERFs (e.g., ERF2) are over 50% identical with ERF1 at the amino acid level [17]. Consistent with the existence of functional redundancy among multiple ERF proteins, the *erf1* and other *erf* single mutants showed no visible phenotype compared with WT in light (Figure S4C). Therefore, we conclude that light increasingly stabilizes ERF1 (and probably other ERF proteins) so that the ERF-mediated hypocotyl inhibition is saturated in strong light conditions, providing an explanation for why ethylene-induced or transgenic ERF1 overexpression functions in dark but not in strong light.

Light has been previously reported to degrade PIFs by activating phytochromes to directly interact with PIFs, and to enhance the accumulation of HY5 by removing COP1/SPA repression [10, 18]. Our study reveals that ERF1 is dramatically stabilized by light irradiation, suggesting that it might represent a new pathway in mediating light-regulated plant development. The relatively slow kinetics of ERF1 protein accumulation upon light irradiation (~2 hr and longer) compared to PIFs (less than 1 hr) implies that photoreceptors are not likely to directly regulate ERF1. Therefore, further studies are needed to examine whether the COP1/SPA complexes modulate ERF1 protein stability and whether the light-induced stabilization of ERF1 and other ERF proteins contributes to the establishment of photomorphogenesis.

We propose a model illustrating how light reverses the function of ethylene in hypocotyl elongation (Figures 4E and S4D). Ethylene constantly activates a promoting pathway dependent on PIF3 and an inhibiting pathway mediated by ERF1. Light progressively destabilizes PIF3 and other PIF proteins but

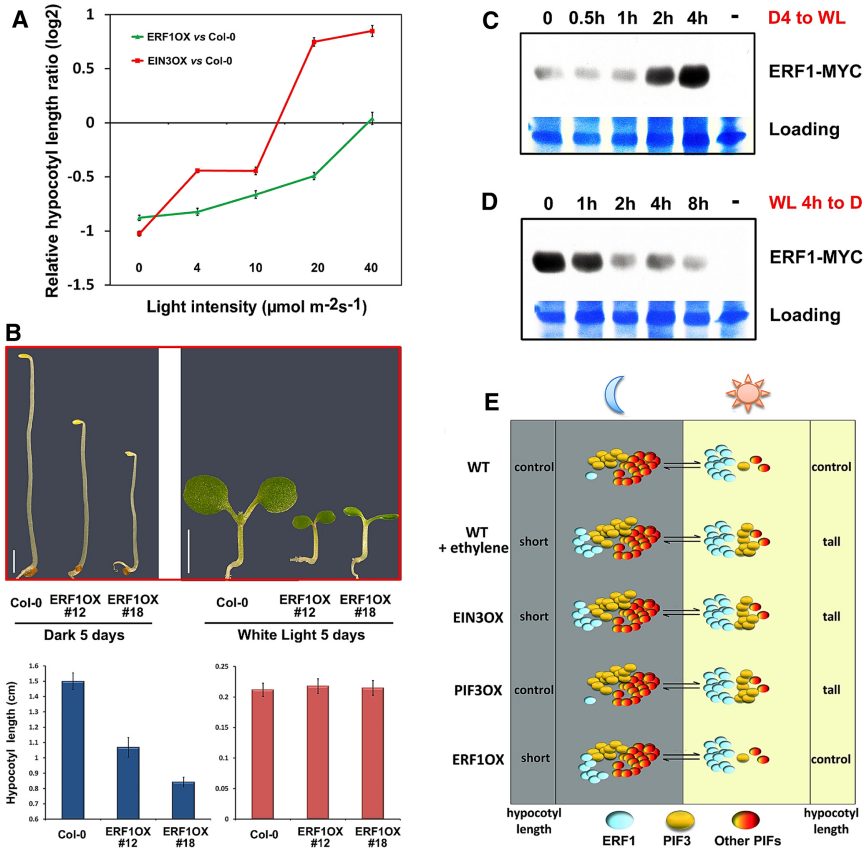

Figure 4. Ethylene Activates an ERF1-Dependent Growth-Inhibition Pathway that Is Progressively Attenuated by Light-Stabilized ERF1 Protein Accumulation

(A) Ratios (in log2) of hypocotyl lengths of 5-day-old seedlings under increasing intensities of continuous light. Data are shown as mean ± SD; n > 20.

(B) Phenotype and hypocotyl lengths of 5-day-old dark-grown or continuous light-grown (40 μmol m^{-2} s^{-1}) seedlings on MS. Data are shown as mean ± SD; n > 20.

(C and D) Immunoblot assay of ERF1 protein (by anti-MYC antibody) in 4-day-old etiolated seedlings constitutively overexpressing ERF1-MYC on MS treated with light irradiation (C) or first illuminated for 4 hr and then returned to darkness (D).

(E) Schematic diagram illustrating the regulation of PIF and ERF1 proteins by light and ethylene. Light induces PIF protein degradation but stabilizes ERF1 protein so that PIF activity is redundant in darkness whereas ERF1 activity is redundant in light.

See also Figure S4.

stabilizes ERF1 protein. As a result, PIF proteins become limited in light but saturated in dark, whereas ERF1 protein is inadequate in dark but abundant in light. Therefore, ethylene-activated PIF3 accumulation is primarily manifested in light to promote hypocotyl elongation, whereas ethylene-induced ERF1 accumulation is mainly functional in dark to repress hypocotyl elongation. Accordingly, overexpression of EIN3 precisely mimics the ethylene effect in both conditions, but overexpression of PIF3 has an effect only in light, whereas overexpression of ERF1 is effective only in dark (Figure 4E).

Interestingly, ethylene has also been reported to activate two functionally opposite transcription factors in rice plants to help survive different types of flooding: ethylene induces SUB1A transcription to restrain rice growth for energy saving during flash flooding [19], whereas under deepwater flooding, ethylene stimulates SNORKEL1/2 transcription to promote stem elongation for keeping leaves above water [20]. It is thus fascinating to explore whether analogous strategies are universally utilized by plants to flexibly and effectively respond to various environmental changes.

Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.039.

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